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Bi-directional regulation of emodin and quercetin on smooth muscle myosin of gizzard

Hou-Li Zhang, Ze-Yao Tang, Jing-Xian Yang, Ying Zhang, Yue Li, Yuan Lin*

Department of Pharmacology, Dalian Medical University, 465 Zhongshan Road, Dalian, Liaoning 116027, PR China

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Abstract **This study is to reveal the characteristics of bidirectional regulation of emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) and quercetin on gizzard smooth muscle myosin. Our results indicate that: (a) emodin demonstrates stimulatory effects, and quercetin produces inhibitory effects on myosin phosphorylation and Mg^{2+}-ATPase activities of Ca^{2+}/calmodulin-dependent phosphorylated myosin in a dose-dependent manner; (b) a combination of emodin and quercetin enhances phosphorylation and Mg^{2+}-ATPase activities for partially phosphorylated myosin and inhibits those activities for fully phosphorylated myosin; (c) 1-(5-Chloronaphthalene-1-sulfonyl)-1H2-hexahydro-1,4-diazepine inhibits myosin phosphorylation in the presence of emodin and/or quercetin. A combination of emodin and quercetin indicates its potential for modulating gastric-intestinal smooth muscle.**

Keywords: Emodin; Quercetin; Myosin Mg^{2+}-ATPase activity; Myosin phosphorylation; Bidirectional regulation

1. Introduction

Emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) is a commonly occurring anthraquinone derivative isolated from Rheum [1], Rheum officinale Baill [2], and other traditional Chinese medicines [3]. Emodin has been reported to significantly inhibit epidermal growth factor (EGF)-induced migration in various human cancer cell lines [4] and to exhibit an immunosuppressive effect [5].

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a flavonoid and is found in various edible plants such as onions, apples, grapes, wine, tea, berries, and spices. Quercetin exerts antioxidant and antihypertensive effects [6]; and at low concentrations, quercetin exhibits anti-proliferative and anti-inflammatory properties [7].

In addition to these effects, the areas of interest are described as follows. (a) Some medicinal herbs contain both emodin and quercetin, e.g., *Aster tataricus* [8], and *Alpinia officinarum* [9]. (b) Both emodin and quercetin can relax vascular smooth muscle [10,11]. (c) Emodin contracts intestinal smooth muscle, and the possible mechanism has been believed to relate to increasing $[Ca^{2+}]_i$ and PKCα translocation, which in turn leads to the activation of myosin and myosin light chain kinase (MLCK) and the suppression of MLCP [12]. Emodin also has been thought to be correlated with the triggering of the release of endogenous acetylcholine, which acts on muscarinic receptors to cause contraction [13]. (d) Quercetin depresses intestinal peristalsis, and this inhibition can be partially prevented by apamin, *N*-nitro-L-arginine methyl ester, and naloxone [14]. Quercetin has also been observed to inhibit intestinal contraction induced by calcium, demonstrating a clear calcium-antagonistic effect [15].

Since the mechanism of smooth muscle contraction and relaxation is related to the regulation of myosin function both directly and indirectly [16,17], the modulation of myosin provides important indexes to evaluate the effects of the modulators. The purpose of our study is to compare the effects of emodin and quercetin on gizzard smooth muscle myosin in a purified system and to reveal the possible interaction between emodin and quercetin on myosin function.

2. Materials and methods

2.1. Materials

1-(5-Chloronaphthalene-1-sulfonyl)-1H2-hexahydro-1,4-diazepine (ML-9), and dithiothreitol (DTT) were purchased from Sigma. Ethyleneglycolbis (2-aminoethy ether) tetraacetic acid (EGTA) was obtained from Wako. Calmodulin (CaM) was generously provided by Pro. K. Kohama, Gumma University, School of Medicine, Japan. Emodin and quercetin were bought from Sigma. Dimethyl sulfoxide (DMSO) was used to prepare 0.1% emodin and 0.1% quercetin stock solution.

2.2. Protein purification

MLCK used in the assay were purified from chicken gizzard smooth muscle as described previously [18]. The purified myosin was unphosphorylated determined by using 10% glycerol electrophoresis.

2.3. Measurement of myosin phosphorylation

Phosphorylation of smooth muscle myosin was carried out in a 20 mmol/l Tris–HCl (pH 7.4) buffer containing 1 mmol/l DTT, 5 mmol/l $MgCl_2$, 60 mmol/l KCl, 0.1 mmol/l $CaCl_2$, 0.6 μmol/l CaM, 4 μmol/l myosin, and 2 mmol/l ATP at 25 °C for 20 min. 10% glycerol polyacrylamide gel electrophoresis (PAGE) was used to measure the extent of phosphorylation of MLC_{20} [19,20].

*Corresponding author. Fax: +86 411 84720652.
E-mail addresses: houlizh@yahoo.com.cn (H.-L. Zhang), liny@dlmedu.edu.cn (Y. Lin)

Abbreviations: MLCK, myosin light chain kinase; CaM, Calmodulin; CDPM, Ca^{2+}/CaM-dependent phosphorylation of myosin; ML-9, 1-(5-chloronaphthalene-1-sulfonyl)-1H2-hexahydro-1,4-diazepine

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2.4. Measurement of myosin Mg^{2+}-ATPase activity

Myosin Mg^{2+}-ATPase activity was measured in a 20 mmol/l Tris–HCl (pH 7.4) buffer containing 60 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l DTT, 0.5 mmol/l ATP, 0.1 mmol/l $CaCl_2$, 0.6 μmol/l CaM, and 0.4 μmol/l myosin at 25 °C for 10 min using the malachite green method [21].

2.5. Other methods

Protein concentration was determined using the Bradford method [22] with bovine serum as standard.

Data were analyzed by ANOVA for multiple data and Student's *t*-test for paired data, using the SSPS software. The level of statistical significance adopted was $P < 0.05$.

To analyze the percentage of MLC_{20} phosphorylation, Scoin Image Software was used to scan the density and size of phosphorylated MLC_{20} and calculate the percentage of phosphorylated MLC_{20} in total MLC_{20}. Mono-phosphorylation was calculated as 100% phosphorylation.

3. Results

3.1. Effects of emodin on myosin Mg^{2+}-ATPase activities

Fig. 1 indicates that emodin at concentrations from 2 to 32 μmol/l induces a dose-dependent stimulation on Ca^{2+}/CaM-dependent phosphorylation of myosin (CDPM), but there are no observable effects on unphosphorylated myosin. With the increase of emodin concentration to 32 μmol/l, the maximal Mg^{2+}-ATPase activity of CDPM reaches 215% compared to the control. In the presence of actin (4 μmol/l), emodin (2–32 μmol/l) also exhibits a dose-dependent stimulation on CDPM and has no observable effects on unphosphorylated myosin (data not shown).

3.2. Effects of quercetin on myosin Mg^{2+}-ATPase activities

Fig. 2 demonstrates that quercetin (2–32 μM) induces a dose-dependent inhibition on Mg^{2+}-ATPase activities of CDPM and has no observable effects on those of unphosphorylated myosin. With the increase of quercetin concentration to 32 μmol/L, the minimum Mg^{2+}-ATPase activity of CDPM myosin is reduced to 51% compared to the control. In the presence of actin (4 μmol/L), quercetin (2–32 μmol/L) also induces a dose-dependent inhibition on Mg^{2+}-ATPase activity of CDPM, and there are no observable effects on unphosphorylated myosin (data not shown).

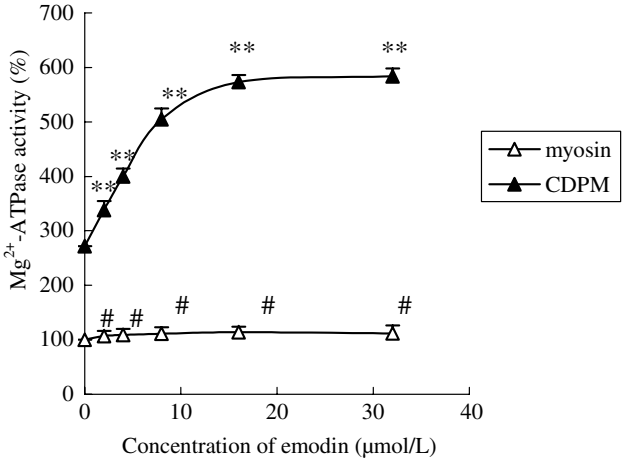

Fig. 1. Effects of emodin in different concentrations on the Mg^{2+}-ATPase activities of CDPM and unphosphorylated myosin. ($\overline{X} \pm s, n = 6$) Filled triangle (▲) represents CDPM, and open triangle (△) represents unphosphorylated myosin. The Mg^{2+}-ATPase activity of unphosphorylated myosin is calculated as 100% $^{**}P < 0.01$, vs. phosphorylated control (without emodin); $^{\#}P > 0.05$, vs. unphosphorylated control (without emodin).

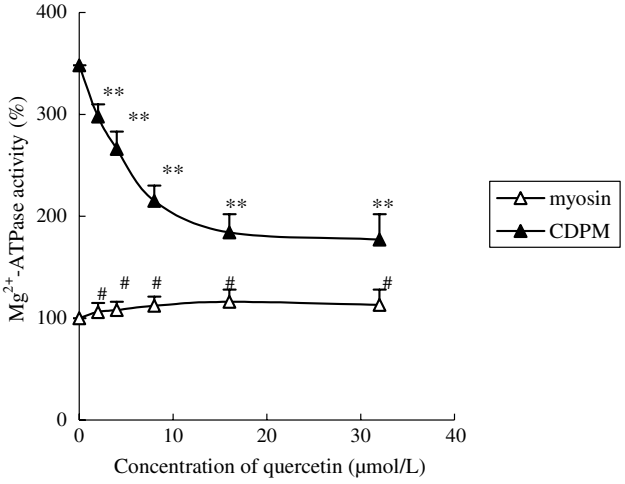

Fig. 2. Effects of quercetin in different concentrations on the Mg^{2+}-ATPase activities of CDPM and unphosphorylated myosin($\overline{X} \pm s, n = 6$). Filled triangle (▲) and open triangle (△) represent CDPM and unphosphorylated myosin respectively. The Mg^{2+}-ATPase activity of unphosphorylated myosin is calculated as 100%. Other data obtained from quercetin are the relative values compared to the corresponding control (without quercetin). $^{**}P < 0.01$, $^{\#}P > 0.05$. Sample with quercetin vs. the corresponding controls without quercetin.

3.3. Interaction of emodin and quercetin on myosin Mg^{2+}-ATPase activities

We have investigated the possible interaction between emodin and quercetin on Mg^{2+}-ATPase activities of CDPM. Using partially phosphorylated myosin, we have observed the following characteristics (Fig. 3A). (a) Both emodin (Column 3) and the combination of emodin and quercetin (Column 4) increase Mg^{2+}-ATPase activities of CDPM, although the potentiation of the latter is weaker than that of the former. (b) The potentiation of Mg^{2+}-ATPase activity by emodin used alone or concurrently with quercetin is inhibited by ML-9 (Columns 5 and 6).

Using fully phosphorylated myosin, we found the following characteristics (Fig. 3B). (a) The concurrent use of emodin and quercetin (Column 4) significantly inhibits the Mg^{2+}-ATPase activities of CDPM, although more weakly than when quercetin is used alone (Column 3). (b) The inhibition of myosin Mg^{2+}-ATPase activity by quercetin and its concurrent use with emodin is further strengthened by ML-9 (Columns 5 and 6).

3.4. Modulation of emodin and quercetin on myosin phosphorylation

We have found that emodin produces a dose-dependent stimulation of myosin phosphorylation at concentrations of 2–32 μmol/l. Quercetin (2–32 μmol/l) induces a dose-dependent inhibition on the extent of myosin phosphorylation (data not shown).

Fig. 4 demonstrates that, for partially phosphorylated myosin, the increase of phosphorylation is observed not only with

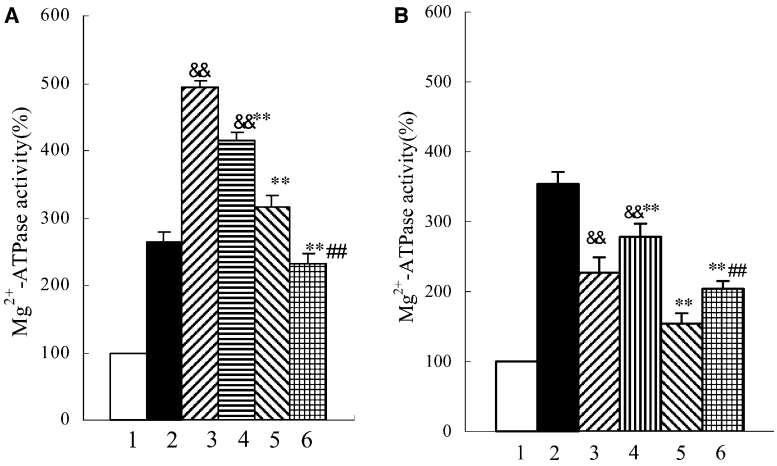

Fig. 3. Effects of emodin and quercetin on the Mg^{2+}-ATPase activities of myosin in different phosphorylation states ($\overline{X} \pm s, n = 6$). (A) Assays are performed using 0.02 μmol/l MLCK and 4 μmol/l partially phosphorylated myosin as described in Section 2; unphosphorylated myosin (Column 1); CDPM (Column 2) ; CDPM + 10 μmol/l emodin (Column 3); CDPM + 10 μmol/l emodin + 10 μmol/l quercetin (Column 4); CDPM + 10 μmol/l emodin + 100 μmol/l ML-9 (Column 5); CDPM + 10 μmol/l emodin + 10 μmol/l quercetin + 100 μmol/l ML-9 (Colomn 6). (B) Assays are performed using 0.2 μmol/l MLCK and 4 μmol/l fully phosphorylated myosin; unphosphorylated myosin (Column 1); CDPM (Column 2); CDPM + 10 μmol/l quercetin (Column 3); CDPM + 10 μmol/l quercetin + 10 μmol/l emodin (Column 4); CDPM + 10 μmol/l quercetin + 100 μmol/l ML-9 (Column 5); CDPM + 10 μmol/l quercetin + 10 μmol/l emodin + 100 μmol/l ML-9 (Column 6); The Mg^{2+}-ATPase activity of unphosphorylated myosin is 100%. The others are the relative values compared to the Mg^{2+}-ATPase activity of unphosphorylated myosin. $^{\&\&}P < 0.01$ vs. Column 2, $^{**}P < 0.01$ vs. Column 3, $^{\#\#}P < 0.01$ vs. Column 4.

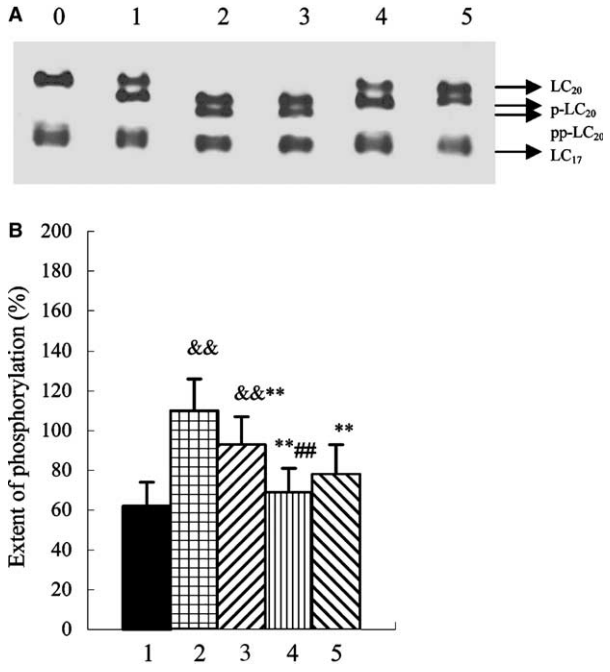

Fig. 4. Effects of emodin and quercetin on partially phosphorylated myosin ($\overline{X} \pm s, n = 6$). 0.02 μmol/l MLCK and 4 μmol/l myosin (partially phosphorylated) are used in the assay. (A) Glycerol PAGE. LC_{20}, unphosphorylated MLC_{20} (20 kDa regulatory myosin light chain); p-LC_{20}, mono-phosphorylated MLC_{20}; pp-LC_{20}, di-phosphorylated MLC_{20}; LC_{17}, 17 kDa myosin essential light chains. Panel A describes: unphosphorylated myosin (Lane 0), CDPM (Lane 1), CDPM + 10 μmol/l emodin (Lane 2), CDPM + 10 μmol/l emodin + 10 μmol/l quercetin (Lane 3), CDPM + 10 μmol/l emodin + 10 μmol/l quercetin + 100 μmol/l ML-9 (Lane 4), and CDPM + 10 μmol/l emodin + 100 μmol/l ML-9 (Lane 5). (B) The extent of myosin phosphorylation, which is analyzed using Scoin Image Software. Mono-phosphorylation is chosen as the control and calculated as 100%, and other data are the relative values compared to the control. $^{\&\&}P < 0.01$ vs. CDPM (Column 1), $^{**}P < 0.01$ vs. CDPM + emodin (Column 2), $^{\#\#}P < 0.01$vs. CDPM + emodin + quercetin (Column3).

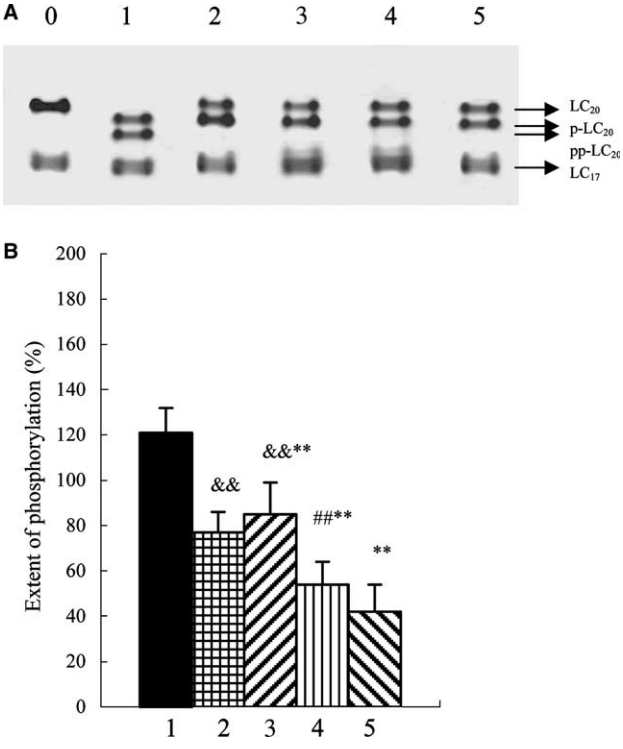

Fig. 5. Effects of emodin and quercetin on fully phosphorylated myosin ($\overline{X} \pm s, n = 6$). The assays were performed using 0.2 μmol/l MLCK and 4 μmol/l myosin (fully phosphorylated). (A) Glycerol electrophoresis results. Unphosphorylated myosin (Lane 0), CDPM (Lane 1), CDPM + 10 μmol/l quercetin (Lane 2), CDPM + 10 μmol/l quercetin + 10 μmol/l emodin (Lane 3), CDPM + 10 μmol/l quercetin + 10 μmol/l emodin + 100 μmol/l ML-9 (Lane 4), CDPM + 10 μmol/l quercetin + 100 μmol/l ML-9 (Lane 5). (B) The extent of myosin phosphorylation, which is analyzed using Scoin Image Software. Mono-phosphorylation is chosen as the control and calculated as 100%. Other data are the relative values compared to the control. $^{\&\&}P < 0.01$ vs. CDPM (Column 1), $^{**}P < 0.01$ vs CDPM + quercetin (Column 2), $^{\#\#}P < 0.01$ vs CDPM + quercetin + emodin (Column 3).

emodin (Lane 2), but also with a combination of emodin and quercetin (Lane 3), and both of the phosphorylation potentiations are inhibited by ML-9 (Lanes 4 and 5). For fully phosphorylated myosin (Fig. 5), not only quercetin (Lane 2) but also the combination of emodin and quercetin (Lane 3) inhibit myosin phosphorylation. Both inhibitory effects are further increased by ML-9 (Lanes 4 and 5).

4. Discussion

Smooth muscle contraction is activated primarily via CDPM by MLCK featured by the enhancement of Ca^{2+} concentration causing the increase of actin-activated myosin ATPase activity, MLC_{20} phosphorylation, and force [23,24]. Since the changes in myosin phosphorylation and Mg^{2+}-ATPase activity reflect the regulation myosin function, we chose them as important indexes to determine the effects of emodin and quercetin on myosin.

Our results demonstrate four characteristics of the regulation of emodin and quercetin on gizzard smooth muscle myosin. (a) Emodin only exhibits stimulatory effects, while quercetin only produces inhibitory effects on Mg^{2+}-ATPase activities of phosphorylated myosin in a dose-dependent manner, regardless of the absence or presence of actin. No observable effects on unphosphorylated myosin are observed for emodin and quercetin. These results differ from some regulatory proteins, e.g., caldesmon and calponin acting on Mg^{2+}-ATPase activity of myosin; Besides activating CDPM, caldesmon and calponin also activate Mg^{2+}-ATPase activity of unphosphorylated myosin to some extent [25,26]. (b) Emodin increases and quercetin decreases the extent of myosin phosphorylation; Such effects are consistent with the effects of emodin and quercetin on Mg^{2+}-ATPase activities of phosphorylated myosin. (c) Concurrent use of emodin and quercetin yields a bi-directional regulation on both Mg^{2+}-ATPase activities of CDPM and the phosphorylation of myosin. Stimulatory effects are observed in partially phosphorylated myosin with 0.02 μmol/l MLCK (Fig. 3A, Column 4, Mg^{2+}-ATPase activity; Fig. 4, Lane 3, Column 3, myosin phosphorylation). Inhibitory effects are obtained for fully phosphorylated myosin using 0.2 μmol/l MLCK, (Fig. 3B, Column 4, Mg^{2+}-ATPase activity; Fig. 5, Lane 3, Column 3, myosin phosphorylation). The bi-directional regulatory effects from emodin and quercetin used in combination imply that gastro-intestinal smooth muscle should not be over contracted or over relaxed if the two agents in combination are properly used. (d) Regardless of the extent of phosphorylation, or whether emodin and quercetin are used alone or in combination, all the effects are inhibited by ML-9, which does not produce bi-directional regulation on myosin in our assay condition.

As we mentioned previously, the consistent effects of emodin and quercetin on vascular smooth muscle, the inconsistent effects on gastro-intestinal smooth muscle, and the co-existence of emodin and quercetin in the same medicinal herbs encouraged us to conduct this investigation. We observed that the concurrent use of emodin and quercetin on various states of myosin phosphorylation produced different responses. These results provide some important information for us to further evaluate the possible mechanism and future clinical application of emodin and quercetin before we offer any conclusions.

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